SAMPLING AND ANALYSIS PLAN FOR THE REGIONAL EXAMINATION OF HARMFUL ALGAL BLOOMS (REHAB)

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1 INTRODUCTION

Toxic cyanobacteria have been detected at a growing rate in western Washington lakes since the first documented toxic episode in American Lake in 1989 (Jacoby et al. 1994, Jacoby and Kann 2007). State health officials are concerned that the rate of occurrence appears to be increasing over time, leading to the possibility of increased human and animal exposure to cyanotoxins. Toxic cyanobacteria blooms are emerging as a national and international environmental and public health issue (Ecology 2007).

Mass accumulations or "blooms" of cyanobacteria in freshwater ecosystems are caused in part by nutrient enrichment. Cyanobacteria blooms can cause surface scums, decreased water column transparency, dissolved oxygen depletion and unpalatable drinking water due to taste and odors. Some cyanobacteria also produce toxic compounds (cyanotoxins) that have caused livestock, wildlife and pet fatalities worldwide (reviewed by Carmichael 1994; Chorus 2001). Although many cyanobacteria blooms are not toxic, a bloom that is not toxic one day may become toxic during the same growing season.

Cyanotoxins include a broad, diverse range of chemicals and mechanisms of toxicity (Carmichael 1994; Sivonen and Jones 1999). Major classes of cyanotoxins include the cyclic peptides, which are primarily hepatotoxins (microcystins and nodularins); alkaloids and an organophosphate, which are strong neurotoxins (anatoxin-a, anatoxin-a (s), and saxitoxins); a cyclic guanide alkaloid, which inhibits protein synthesis (cylindrospermopsin); lipopolysaccharides, which have pyrogenic properties; and dermatoxic alkaloids (aplysiatoxins and lyngbyatoxins) (Table 1) (Chorus 2001). This REHAB SAP will focus on four toxins: microcystins, anatoxin-a, saxitoxins and cylindrospermopsin.

Microcystins are the most commonly tested and detected cyanotoxins in Washington and have been found at water concentrations from <1 to 4,810 µg/L (Jacoby and Kann 2007). These hepatotoxins are of concern due to their prevalence and potential to harm animals and humans. Hepatotoxins damage liver tissues, and at high doses can cause liver failure and death (Carmichael 1994). Microcystins have seven amino acids and are produced by species of *Microcystis, Planktothrix,* and *Anabaena* and *Glocotrichia*. The mechanism of toxicity involves inhibition of the specific protein phosphatase enzymes possessed by all eukaryotic cells. In addition, microcystins are suspected tumor-promoters and teratogens (Falconer 1998). These toxins have been associated with elevated rates of primary liver cancer in people drinking waters with high densities of cyanobacteria (Yu 1989). Nodularin, also a hepatotoxin, has five amino acids and is produced by *Nodrularin spumigena*.

While microcystins appear to be more common than neurotoxins, neurotoxins are notoriously potent and rapid acting poisons that have caused severe animal poisonings in North America, Europe and Australia (WHO 2003, Botana 2007). The neurotoxin anatoxin-a is an alkaloid with high toxicity. It acts as a post-synaptic, depolarizing, neuromuscular blocking agent. Depending upon the size of the animal and amount of the

Table 1. General features of cyanotoxins (modified from Chorus and Bartram 1999).		
Toxin Group	**Primary Target organ in mammals**	**Cyanobacterial genera[2]**
Microcystins[1]	Liver	*Microcystis, Anabaena, Planktothrix (Oscillatoria), Nostoc, Hapalosiphon, Anabaenopsis*
Nodularian	Liver	*Nodularia*
Anatoxin-a	Nerve Synapse	*Anabaena, Planktothrix (Oscillatoria), Aphanizomenon*
Anatoxin-a (s)	Nerve Synapse	*Anabaena*
Aplysiatoxins	Skin	*Lyngbya, Schizothrix, Planktothrix (Oscillatoria)*
Cylindrospermopsins	Liver	*Cylindrospermopsis, Aphanizomenon*
Lyngbyatoxin-a	Skin, G.I. Tract	*Lyngbya*
Saxitoxins[1]	Nerve Axons	*Anabaena, Aphanizomenon, Lyngbya, Cylindrospermopsis*
Lipopolysaccharide	Potential irritant; affects any exposed tissue	ALL
BMAA	Neurodegerative	*Nostoc*

1. Many structural variants may be known for each toxin group.
2. Not produced by all species of a particular genus
3. Whole cells of toxic species elicit widespread tissue damage to kidney and lymphoid tissue.

toxin present, illness or death may occur within a minutes to a few hours after exposure. Signs of anatoxin-a poisoning are staggering, paralysis, muscle twitching, gasping, and convulsions – all potentially leading to death. Anatoxin-a can be produced by some species of cyanobacteria including *Anabaena*, *Aphanizomenon, Cylindrospermum*, *Planktothrix* (*Oscillatoria), and Microcystis* spp.. Determination of anatoxin-a in natural waters has been challenging due to its typically low concentration and/or rapid degradation to nontoxic degradation products (Botana 2007).

Cylindrospermopsin is a cyclic guanidine alkaloid hepatotoxin with a molecular weight of 415 first identified in *Cylindrospermopsis raciborskii* and most commonly observed in tropical and subtropical waters of Australia (Ohtani 1992, Fastner 2003). It is a potent inhibitor of protein synthesis and is found in three variants and five genera (*Cylindrospermopsis, Anabaena, Raphidiopsis, Aphanizomenon*, and *Umezekia*) (Schembri et al. 2001). It has been increasingly encountered in temperate regions and has caused blooms as far north as Vienna and northeastern Germany. New structural variants have been isolated recently from an Australian strain of *C. raciborskii* (Chiswell 2001).

The first report of animal poisonings attributed to *C. raciborskii* and cylindrospermopsin was by Saker et al. (1999) in drinking water in a farm pond in Queensland, Australia, where it was responsible for cattle deaths. Cylindrospermopsin was identified as the probable cause of

extensive poisoning through drinking water of 138 children and 10 adults in 1979 known as the Palm Island mystery disease. Cylindrospermopsin has been identified in drinking water sources in the U.S., Europe, Israel, Brazil, Southeast Asia, Japan and Australia. In pure form, it mainly affects the liver, although crude extracts of *C. racinborskii* injected or given orally to mice induce pathological symptoms in the kidneys spleen, thymus and heart (Botana 2007). Symptoms of liver toxicosis include nausea, vomiting, and acute liver failure. Clinical symptoms after exposure to cylindrospermopsin may manifest only several days after exposure (WHO 2003). Laboratory studies have shown that some of the compounds produced by *Cylindrospermopsis* may be carcinogenic and genotoxic.

C. raciborskii does not form scums and has the highest cell concentrations well below the water surface. Unlike *Microcystis*, *C. raciborskii* appears to release toxin into the water during growth (Shaw et al. 1999). Cylindrospermopsin is relatively stable in the dark. Breakdown occurs slowly at elevated temperatures (50^{o}C) but quite rapidly in the sunlight and in the presence of cell pigments, with more than 90% completion within 2-3 days. However, pure cylindrospermopsin is relatively stable in sunlight. (Chiswell et al. 1999).

Saxitoxin is another neurotoxin produced by blue-green algae and is a carbamate alkaloid sodium channel blocker. Saxitoxins have been observed in *Aphanizomenon, Anabaena, Lyngbya, Cylindrospermopsis*, and *Planktothrix.* Twenty variants have been identified (Huisman et al.). Animals with saxitoxin/neosaxitoxin toxicosis may exhibit weakness, staggering, loss of muscle coordination, difficulty in swallowing, labored respiration, complete muscle paralysis, and death. Humans may exhibit tingling around the mouth and fingertips, as well as slurred speech. Nearly all systemic effects of saxitoxin are due to its effect on nerve axon membranes. Saxitoxins can be produced by several species of cyanobacteria including *Anabaena*, *Aphanizomenon, Cylindrospermopsis*, and *Lyngbya*. Saxitoxins from *Anabaena circinalis* may bioaccumulate in an Australian mussel to levels exceeding human health international guidelines in as few as seven days (Shumway et al. 1995).

Saxitoxins and anatoxin-a(s) are among the most neurotoxic substances known (WHO 2003). They do not occur in lakes and rivers as frequently as microcystins. After ingestion of a sublethal dose of neurotoxins produced by cyanobacteria, recovery appears to be complete. Chronic effects have not been observed. Neurotoxins have the potential to be lethal by causing suffocation, specifically through paralysis by exposure to saxitoxins. No human deaths from exposure to neurotoxins associated with recreational use of water are known.

Saxitoxins undergo a series of slow chemical hydrolysis reactions in the dark at room temperature. Half-lives for breakdown reactions are in the order of 1 – 10 weeks, with more than 3 months required for greater than 90% breakdown. In some circumstances, breakdown of a bloom will increase the toxicity over a period of up to three weeks before toxicity abates in the following 2-3 months, since some breakdown products are much more toxic than the intact toxin. Boiling an extract of *Anabaena* may also increase toxicity. No detailed studies have been carried out on saxitoxin breakdown in sunlight with or without pigments (Chorus and Bartram 1999).

Highest published concentrations from cyanobacterial bloom samples measured by HPLC are 5,500 ug/g dw cylindrospermopsin (Australia), 3,400 ug/g dw saxitoxin (Australia), 7,300 ug/g dw microcystins (China, Portugal); anatoxin-a 4,400 ug/g dw (Finland) (Chorus and Bartram 1999).

1.1 Project Background

Cyanobacterial toxins produced by harmful algal blooms (HABs) are a worldwide public health threat. Health effects from cyanobacteria exposure in recreational waters are diverse and include skin rashes and lesions, vomiting, gastroenteritis, conjunctivitis, headaches, and eye, ear and throat irritations (Pilotta *et al.* 1997, Chorus *et al.* 2000, Codd *et al.* 2005). While many of these reactions have been documented, information on cyanobacterial species, cell densities, and toxins present during exposure is typically lacking (Chorus 2005, Codd *et al.* 2005). Attempts to characterize human health hazards during primary contact activities have been based on limited human case histories, reported animal poisonings, and human epidemiological data (Pilotto *et al.* 1997).

Toxic cyanobacterial blooms have been documented in numerous Washington State lakes during the past 30 years (Jacoby and Kann 2007). The first documented health effects of cyanotoxins in Washington State occurred in the 1970s and 1980s, causing the deaths of several dogs. More recently, animal deaths have been associated with the detection of anatoxin-a in several western Washington lakes (*e.g.*, American Lake, Pierce County, December 1989 and 2007; Kitsap Lake, Kitsap County, October 2001; and Anderson Lake, Jefferson County, June 2006). Over the past decade, several anecdotal reports have been received of human illness, such as gastrointestinal symptoms and rashes, following recreational exposure to blooms. A particularly toxic bloom of *Microcystis* occurred in Lake Sammamish in 1997 and was believed to have caused illnesses of children swimming in the lake and the death of a dog (Johnston and Jacoby 2004).

Following the aforementioned animal deaths, Washington's legislature funded the Freshwater Algae Control Program, implemented by Washington State Department of Ecology (Ecology). As part of this program, local jurisdictions and citizens can submit bloom samples for state-funded analysis of microcystin concentrations and phytoplankton composition. In partnership with Ecology, Washington State Department of Health (DOH) is in the initial phase of working with jurisdictions statewide to educate agencies and citizens on bloom identification and on public health effects of HABs. This partnership has set a foundation for cooperative work in the state.

The U.S. Centers for Disease Control and Prevention (CDC) has funded a proposal entitled "Regional Examination of Harmful Algal Blooms" (REHAB) with the following goals:

1. Incorporate existing Washington HAB data into CDCs Harmful Algal Bloom-related Illness Surveillance System (HABISS);
2. Expand and standardize HAB reporting and monitoring at a regional scale to improve the understanding of HAB frequency and associated risks;
3. Investigate the possible occurrence and health risks from freshwater recreational exposure to the algal toxins saxitoxin and cylindrospermopsin in this region;
4. Develop statewide recreational standards for saxitoxin and cylindrospermopsin, if detected, and
5. Increase and refine public outreach to improve awareness of HABs and provide effective methods for public health notification and prevention activities.

REHAB will be administered by DOH in partnership with Ecology; Pierce, King and Snohomish Counties; and Seattle University (SU).

1.2 Regulatory Status of Cyanotoxin Criteria and Guidelines

In 2005, the Washington State Legislature established funding for a Freshwater Algae Control Program (RCW 43.21A.667) through the Department of Ecology (Ecology) to assist local governments in the management of freshwater algae problems. As part of this program Ecology partnered with the Washington Department of Health (DOH) to develop recreational guidelines values for cyanotoxins. DOH has recommended a three-tiered approach using the recreational guidance values of 6.0 µg/L microcystins and 1 µg/L anatoxin-a for managing Washington Lakes (DOH, 2008). More information about this three-tiered management approach can be found at the Washington State Department of Health webpage: http://www.doh.wa.gov/ehp/algae/guidelines.htm.

1.3 Study Area Description

Ten lakes were selected by each County based on public access, history of blue-green algae blooms and potential toxicity as well as help from volunteers who live on the lake (Table 2 and Figure 1). Routine monitoring will occur at each of these lakes twice a month from June through October at the surface and at the most heavily used area by citizens. Wind often blows algae to one side of a lake, forming a "scum" that will be also sampled as they occur. Most samples will be collected by volunteers identified by County staff.

Table 2. Stations and Sampling Parameters at Each Location.

County	Station	Locator	Parameter1	Annual # samples/
King	Beaver Lake	CDC100-A709	M/H/Q/A/S/C	10
King	Cottage*	CDC100-A707	M/H/Q/A/S/C	10
King	Echo	CDC100-A764	M/H/Q/A/S/C	10
King	Lake Desire	CDC100-A711	M/H/Q/A/S/C	10
King	Lake Marcel	CDC100-A765	M/H/Q/A/S/C	10
King	Lake Sawyer	CDC100-A718	M/H/Q/A/S/C	10
King	Lake Wilderness*	CDC100-A717	M/H/Q/A/S/C	10
King	Shadow Lake	CDC100-A714	M/H/Q/A/S/C	10
King	Spring Lake	CDC100-A712	M/H/Q/A/S/C	10
King	Tuck Lake	CDC100-A760	M/H/Q/A/S/C	10
Pierce	American Lake	CDC200-1	M/H/Q/A/S/C	10
Pierce	Bay Lake	CDC200-2	M/H/Q/A/S/C	10
Pierce	Clear Lake	CDC200-3	M/H/Q/A/S/C	10
Pierce	Harts Lake	CDC200-4	M/H/Q/A/S/C	10
Pierce	Ohop Lake	CDC200-5	M/H/Q/A/S/C	10
Pierce	Spanaway Lake	CDC200-7	M/H/Q/A/S/C	10
Pierce	Steilacoom Lake	CDC200-6	M/H/Q/A/S/C	10
Pierce	Tanwax Lake	CDC200-8	M/H/Q/A/S/C	10
Pierce	Silver Lake *	CDC200-10	M/H/Q/A/S/C	10
Pierce	Waughop Lake *	CDC200-9	M/H/Q/A/S/C	10
Snohomish	Armstrong	CDC300-4	M/H/Q/A/S/C	10
Snohomish	Blackman	CDC300-8	M/H/Q/A/S/C	10
Snohomish	Cassidy *	CDC300-7	M/H/Q/A/S/C	10
Snohomish	Chain	CDC300-9	M/H/Q/A/S/C	10
Snohomish	Ketchum *	CDC300-2	M/H/Q/A/S/C	10
Snohomish	Loma	CDC300-6	M/H/Q/A/S/C	10
Snohomish	Lost	CDC300-1	M/H/Q/A/S/C	10
Snohomish	Martha	CDC300-5	M/H/Q/A/S/C	10
Snohomish	Stevens	CDC300-10	M/H/Q/A/S/C	10
Snohomish	Sunday	CDC300-3	M/H/Q/A/S/C	10

* Stations that will be sampled for saxitoxin and cylindrospermopsin in 2009, all others will be sampled in 2010.

PARAMETER CODES:
M = microcystin analysis
Q = quantitative phytoplankton analysis routinely done
A = anatoxin-a analysis
S = saxitoxin analysis
C = cylindrospermopsin analysis

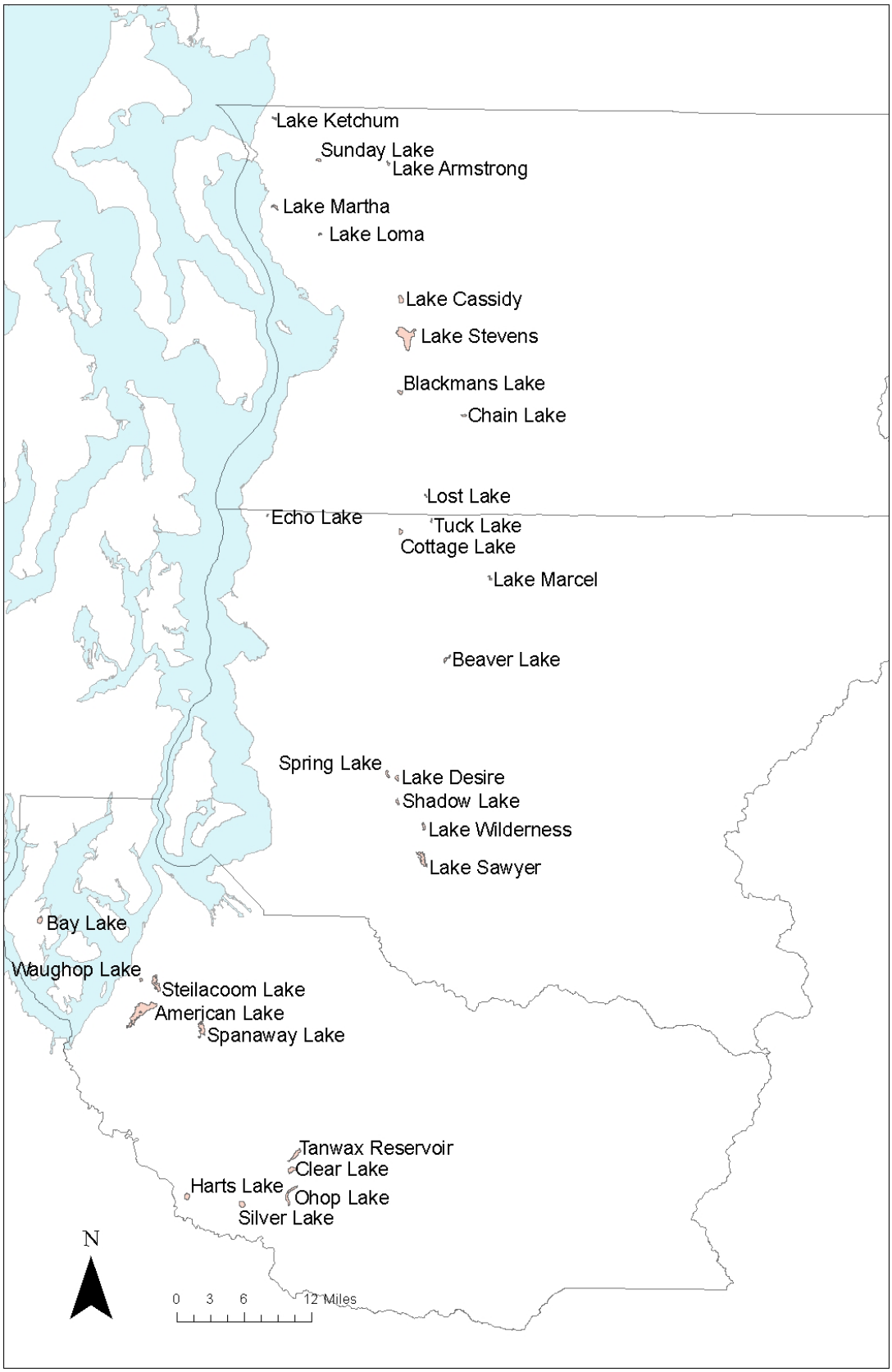

Figure 1: Location of 30 lakes participating in REHAB Project in the three county area, Washington.

1.4 Project Objectives

Objectives of the REHAB project are to:

a. Expand regional monitoring in collaboration with programs in three counties to obtain environmental data associated with toxic blooms (for HABISS).

b. Test for microcystin and anatoxin-a toxicity with more regularity in regional lakes, and develop a reproducible, systematic monitoring program for cyanobacterial toxicity measurements.

c. Investigate the presence of two cyanotoxins previously undetected in Washington: cylindrospermopsin and saxitoxins.

d. Identify phytoplankton collected during monitoring efforts.

The study will provide decision-makers with information and recommendations regarding recreational water use during cyanobacterial blooms and will lead to improved management of state lakes for the protection of human health.

2 STUDY DESIGN

2.1 Approach

REHAB is designed to develop and implement a reproducible, systematic monitoring program for cyanobacterial toxicity measurements in the heavily populated Puget Sound lowland region, specifically 30 lakes in Snohomish, King and Pierce Counties. Monitoring efforts will begin in June of 2009.

Sample collection will utilize the combined efforts of agencies from three counties: King County Water and Land Resources Division, Snohomish County Public Works and Tacoma/Pierce County Public Health. Each county will select ten lakes based on potential to produce toxic blue-green algae blooms as well as public access and use.

The 30 selected regional lakes will be sampled twice a month from June – October. Samples will be collected at a routine sampling spot, preferably at the surface where there is public use/access. Samples will be collected by identified and trained volunteers on as many lakes as possible. If there is visible scum present elsewhere along the lake shoreline during the routine sampling event, a second sample will be collected at that location (up to 15 scum samples beyond the routine 300 samples for all lakes). If scum samples are present above and beyond the 15 scum samples allowed through the REHAB project, the Washington Department of Ecology (DOE) Algae program has agreed to test extra samples as they occur.

Quantitative phytoplankton samples will be collected at each site for a total of 30 lakes (Table 2). Phytoplankton samples will be sent to a contractor for identification and enumeration.

The following three types of sampling scenarios are included in this study:

1. Routine Lake Sampling. Thirty lake shoreline sampling sites were selected for the REHAB project. Routine monitoring will implement a consistent monitoring protocol across the

region to answer the question whether the frequency of known and novel cyanotoxins in regional lakes is higher than indicated by limited monitoring for these substances to date.

This effort will sample (on a routine basis, twice a month June through October) lakes for cyanotoxicity by tracking the presence of cyanotoxins, evaluating cell densities and types of cyanobacteria during toxic events, determining the percentage of sampling events where toxins were produced, and determining the number of events that pose a potential public health threat. Regular monitoring of selected lakes known to produce toxic blooms will allow a timelier public health response as well as a more accurate assessment of cell counts of cyanobacteria that can produce health issues.

- Microcystins will be measured by Enzyme Linked Immunosorbent Assay (ELISA) at the King County Environmental Lab
- Anatoxin-a will be measured by High Performance Liquid Chromatography with Fluorescence Detection at the King County Environmental Lab

2. Additional Toxin Sampling. Sampling will include investigation of the presence and potential health risks of saxitoxins and cylindrospermopsin throughout the region. Saxitoxins may be present based on observed high cell volumes of cyanobacteria known to produce saxitoxins. Cylindrospermopsin has not been observed in the region. It is unknown at this time if either toxin is present in Puget Sound Lowland lakes. This study will further define the level of potential threat by cyanotoxin production in Washington State.

   - Saxitoxins will be measured by Enzyme Linked Immunosorbent Assay (ELISA) at the King County Environmental Lab
   - Cylindrospermopsins will be measured by Enzyme Linked Immunosorbent Assay (ELISA) at the King County Environmental Lab

3. Cyanobacteria bloom sampling. Focused sampling efforts will be made to collect blooms or accumulations of cyanobacteria if they are present within the visual distance of routine lakes sampling sites. A bloom will be defined by a visually observable accumulation of phytoplankton in the water column or as a surface accumulation. Coordinates will be obtained for these grab samples and a LIMS locator created. New locator names will be consistent with the naming convention system established for the REHAB project.

   Up to 15 samples above and beyond the routine 300 samples may be collected throughout the sampling season for blooms. If other blooms occur during the season, the Ecology's Freshwater Algae Control Program has agreed to run sample analyse for the bloom.

2.2 Timeline

The REHAB Sampling Analysis Plan (SAP) will be implemented from June 2009 and continue through October 2011. Microcystins and anatoxin-a will be sampled routinely. In 2009, saxitoxin and cylindrospermopsin will be sampled on a limited basis (2 locations in each county twice a month) to refine sample collection, preparation, dilution and analysis procedures. It is expected that saxitoxin and cylindrospermopsin analysis will become a regular part of cyanotoxin monitoring for all stations in 2010. In 2010, the program will be reevaluated and modifications made as necessary.

2.3 Sampling Procedures

Methods for the extraction and analysis of cyanotoxins are evolving rapidly and currently are not consistent among laboratories (Grahm, J. 2008). The following sampling procedures are based on methods of Carmichael (2001), Chorus (2001), Johnston and Jacoby (2002) (Table 3).

Table 3. Sample Container & Preservation Requirements

Parameter	Matrix	Container	Preservation	Hold time
Quantitative Phytoplankton	Liquid	1x 250- mL Plastic Amber Wide Mouth (AWM)	Lugol's solution pre-preserved Store at room temperature in the dark	365 days
Microcystins ELISA	Liquid	250- mL Glass, AWM	4°C, Dark	24 to 48 hours to freeze, 14 days for analysis
Cylindrospermopsin ELISA	Liquid	250- mL Glass, AWM (same bottle as collected for MLR-ELISA)	4°C, Dark	24 to 48 hours to freeze, 14 days for analysis
Saxitoxin ELISA	Liquid	Amber VOA vial	In the field a specified volume of sample is added to a sample bottle containing preservative in the ratio of 1:10 preserv.:sample 4°C, Dark	7 days with preservation, up to 14 days if frozen for analysis
Anatoxin-a HPLC	Liquid	2x 1– L Plastic Amber bottle (2 per station)	4°C, Dark	24 to 48 hours for filtration/SPE 28 days for analysis

Notes: AWM – Amber wide mouth bottle

2.3.1 Water sample collection and storage procedure to test for toxins:

Samples will be collected using the site-specific collection method identified above in Section 2.1 (e.g., surface grab).

For surface grabs, fill the 250 mL glass, AWM bottle and the 2 1-L plastic amber bottles by dipping the bottle mouth-down into the water. With a sweeping arch, collect water from approximately 1 ½ feet below the surface, leaving some headspace to allow for freezing. An 18 mL portion of the sample from the 250 mL AWM bottle will be pipetted into the 40 mL VOA vial for saxitoxin analysis.

Other sampling protocol notes:

- Label the bottles if not pre-labeled
- Place the sample bottles for microcystins, cylindrospermopsin and anatoxin-a directly in a cooler with ice packs (no preservative required)
- For saxitoxin, a measured 18 mL of sample is added to the sample vial containing preservative. The sample container is next inverted several times for mixing, placed in a cooler with ice packs
- Phytoplankton samples will be collected in 250 mL and preserved with 1.75 mL of Lugol's solution. The solution will be put in the sample within 24 hours of collection
- Sub-samples will be removed from the 250 mL glass bottle and frozen within 24 to 48 hours of arrival at the KCEL. Bottles and vials should be slanted to prevent breakage during freezing. Samples must be stored frozen for a minimum of 12 hours to insure complete freezing of the sample

2.3.2 Water sample collection and storage procedure for quantitative identification of cyanobacteria.

Phytoplankton samples will be collected using the site-specific collection method identified above in Section 2.1 (following routine lake and cyanobacteria algal bloom sampling scenarios).

All routine phytoplankton samples will be collected in a 250 mL plastic wide-mouth bottle, appropriately labeled and preserved with a sufficient amount of concentrated Lugol's solution to turn the sample light orange-red (typically 1.75 mL Lugol's). Care should be taken that sample bottles are covered tightly and stored in a cool, dark place (i.e., refrigerator) until sent to contractor for analyses. Quantitative phytoplankton identification and enumeration of cyanobacteria and non-cyanobacteria groups will be conducted by WATER Environmental Services, Inc.

As a cost saving measure, the first June 2009 routine sampling event (30 total samples) will result in a semi-quantitative analysis for cyanobacteria taxa only (CYANO ONLY TAXA) by WATER Environmental Services, Inc. These first June samples will be stored to allow for more in-depth analysis later, if needed.

In the event that additional algal blooms are sampled in scum conditions, up to 15 bloom samples will be collected, preserved, and analyzed by WATER Environmental Service, Inc.

under the REHAB program, as described above. Additional scum samples collected beyond the 15 bloom samples will follow the protocol of the Washington Department of Ecology's Freshwater Algae Program.

3 LABORATORY ANALYSIS

Enzyme Linked Immunosorbent Assays (ELISA) and assays are suitable for rapid and sensitive detection of algal toxins. These methods are useful for preliminary toxin screening of both cyanobacterial samples and extra cellular toxins in the water (Chu et al. 1990; Chorus 2001). ELISA sensitivity is based on the structure of the toxin molecule and requires antibodies against the toxin of interest.

3.1 Sample Preparation for Algal Toxin ELISA

Samples will be analyzed for total microcystins, cylindrospermopsin, and saxitoxin using ELISA. To measure total microcystin and cylindrospermopsin concentrations (extra- and intracellular) in the water samples, sample preparation will include a cell lysing step prior to analysis. Saxitoxin analysis requires preservation (preservative provided in the ELISA kit) of the sample in the field prior to analysis and will not include a cell lysing step.

3.1.1 Cell Lysing Procedure for Microcystins and Cylindrospermopsin

The objective of the cell lysing is to generate a sample in which all microcystin or cylindrospermopsin (extra- and intracellular) has been converted into a free form that can be measured by ELISA, thus providing a close approximation of the total concentration in the ambient sample (extra- plus intracellular). The resulting concentration should be representative of a recreational exposure in which a swimmer ingests ambient water and cells as a combined dose. If samples were analyzed without lysing, results would be reported as *Extracellular.* Since all samples collected for this study will be analyzed following lysing, results will be reported as *Total Microcystins and Total Cylindrospermopsin*. Note that ELISA generally measures only free analytes, not the amount chemically bound to the cell or other molecular components.

Based on previous cyanobacteria toxin work done, laboratory staff recommended combining extraction methods. Therefore, each sample will be prepared for analysis by the following lysing process:

- 10-mL aliquots will be frozen for a minimum of 12 hours
- Frozen samples will be thawed at room temperature and then immediately sonicated (ultrasonic disruption) using the Vibra Cell Sonicator
- Samples will be filtered through a 0.45 μm filter prior to analysis

3.1.2 Sample Preparation: Preservation Procedure for Saxitoxin

A 10x Concentrated Sample Diluent is provided in the Abraxis Saxitoxin ELISA kit. The sample container will have the Sample Diluent added prior to being taken into the field and it will be necessary to add a specific volume of sample in the ratio of 1:10 (diluent:sample). In this case, 2 mL of sample diluent will be added to a 40 mL sample container and in the field 18 mL

of sample must be pipetted (alternately, a graduated cylinder can be used for thick cellular material) to the sample vial. The sample container is then inverted several times for mixing. Note: If a lab or field duplicate is being done for the sampling event, a second vial should be prepared in this same manner to be used for lab QC.

3.1.3 Microcystins– ELISA

The Beacon ELISA test kit uses polyclonal antibodies that bind either microcystins or a microcystin-enzyme conjugate. Microcystins in the sample compete with the microcystin-enzyme conjugate for a limited number of antibody binding sites. Since the same number of antibody binding sites are available on every test well, and each test well receives the same number of microcystin-enzyme conjugate molecules, a sample that contains a low concentration of microcystins allows the antibody to bind many microcystin-enzyme conjugate molecules. The result is a dark blue solution. Conversely, a high concentration of microcystins allows fewer microcystin-enzyme conjugate molecules to be bound by the antibodies, resulting in a lighter blue solution. The plate kit does not differentiate between microcystin-LR and other microcystin variants but detects their presence to differing degrees. At 50% inhibition the concentrations are: MC-LR 0.31 μg/L, MC-RR 0.32 μg/L, MC-YR 0.38 μg/L and NODLN 0.47 μg/L. The microcystin assay has an operational range of 0.16 to 2.5 ug/L. Samples with values less than 0.16 will be reported as less than MDL, samples above 2.5 will be diluted and re-run to give a reportable value from within the standard curve.

3.1.4 Cylindrospermopsin ELISA

The Abraxis test is a direct competitive ELISA that allows the detection of cylindrospermopsin. It is based on the recognition of cylindrospermopsin by specific antibodies. Cylindrospermopsin, when present in a sample, and a cylindrospermopsin-HRP analogue compete for the binding sites of rabbit anti-cylindrospermopsin antibodies in solution. The cylindrospermopsin antibodies are then bound by a second antibody (sheep anti-rabbit) immobilized in the plate. After a washing step and addition of the substrate solution, a color signal is generated. The intensity of the blue color is inversely proportional to the concentration of the cylindrospermopsin present in the sample. The color reaction is stopped after a specified time and the color is evaluated using an ELISA Plate reader. Sample concentrations are determined by interpolation using the standard curve constructed with each run. The cylindrospermopsin assay has an operational range of 0.05 to 2.0 ug/L. Samples with values less than 0.05 will be reported as less than MDL, samples above 2.0 will be diluted and re-run to give a reportable value from within the standard curve.

3.1.5 Saxitoxin ELISA

The test is a direct competitive ELISA based on the recognition of saxitoxins by specific antibodies. Saxitoxins, when present in a sample and a saxitoxin-enzyme-conjugate compete for the binding sites of rabbit anti-saxitoxin antibodies in solution. The saxitoxin antibodies are then bound by a second antibody (sheep anti-rabbit) immobilized on the plate. After a washing step and addition of the substrate solution, a color signal is produced. The intensity of the blue color is inversely proportional to the concentration of the saxitoxin present in the sample. The color reaction is stopped after a specified time and the color is evaluated using an ELISA reader. Concentrations of the samples are determined by interpolation using the standard curve constructed with each run. The saxitoxin assay has an operational range of 0.02 to 0.4 ug/L. Samples with values less than 0.02 will be reported as less than MDL, samples above 0.4 will be diluted and re-run to give a reportable value from within the standard curve.

3.2 Anatoxin-a

The neurotoxin anatoxin-a is an alkaloid with high toxicity (LD_{50} i.p. mouse 200 μg/kg). It acts as a post-synaptic, depolarizing, neuromuscular blocking agent. Anatoxin-a is produced by some species of *Anabaena*, *Aphanizomenon, Cylindrospermum* and *Oscillatoria*.

The determination of anatoxin-a in natural waters has been challenging due to its typically low concentration. The highly sensitive method adapted at KCEL uses fluorimetric derivatization with HPLC. Following extraction with methanol, anatoxin-a is converted into a fluorescent derivative using 4-fluoro-7-nitro-2,1,3-benzoxadiazole; the fluorescent compound is then separated and detected by HPLC. Increased sensitivity is accomplished by previously concentrating the sample and the extract.

3.2.1. Sample Handling and Preparation

Anatoxin-a degrades readily, especially in sunlight and at high pH. At KCEL the bottles are immediately transferred to a 4 °C chamber. Samples are frozen whole (Solid Phase Extraction, SPE, method) or filtered onto glass fiber filters (particulate method) within 24 hours of arrival and the filters stored in cryovials at -20 °C.

Two alternative sample concentration and extraction methods are available. The most suitable method will be used after the sample has been evaluated. Solid Phase Extraction (SPE) of whole water samples may be the preferred method for samples with high biomass (i.e., blooms), but may also be used for samples with very low biomass, whereas an extraction of the particulate fraction may be preferred for samples with low biomass, as the latter method makes it possible to concentrate a large volume of sample for extraction. Because the particulate extraction method only measures intracellular toxin it may underestimate the total amount of toxin present in the environment, especially in senescing populations. However, because anatoxin-a may be easily degraded once outside the cell in the photic zone, ambient concentrations of active extracellular toxin are most likely very low in healthy populations.

Cells are ruptured by freezing and sonication and anatoxin-a is extracted with acidified methanol. The resulting extract is evaporated under nitrogen and stored at -20 °C.

Dried samples or standards are derivatized with 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F). The reaction is terminated at 60 minutes, and samples are filtered and transferred to autosampler vials.

3.2.2. Chromatography

HPLC analysis is performed with an Agilent 1200 series system using a Zorbax C_{18} column, 45% acetonitrile-phosphate buffer as the mobile phase and fluorimetric detection at 470 nm / 530 nm. Run time is 37 min per injection.

Anatoxin-a analytical standards are purchased commercially, diluted and run along with samples to create a 4-point calibration curve. Methyl pipecolinate is added to all samples and standards and used as a reference standard.

3.2.3 Method Detection Limits

Because samples are concentrated prior to chromatography, each sample carries a sample-specific concentration factor (CF) and therefore the final MDL is also sample-specific. The concentration factor is based on the volume of water extracted by SPE or filtered for cell extraction, the volume of extract dried, and the final volume of the derivatized extract.

MDL, SPE method: The current Calculated MDL is 8 µg/L
The final MDL for each SPE sample is:

Final MDL = (Calculated MDL) / CF = (8 µg/L)/CF

MDL, Particulate method: The current Calculated MDL is 1.3 µg/L, but the Reported MDL has been set at 5 µg/L to account for background interference in natural samples.

The final MDL for each particulate sample is:

Final MDL = (Reported MDL) / CF = (5 µg/L)/CF

For example, the final sample MDL for a 2 L sample extracted in 10 mL methanol, 3 mL of extract dried and derivatized to a final volume of 400 µL is: Final MDL = (5 µg/L) / (200 x 7.5) = 0.0033 µg/L.

Strong matrix effects could significantly affect the level of detection for certain samples.

3.3 Analytical Procedures

Samples will be analyzed using the procedures and detection limits listed in table 4.

Table 4. Laboratory Analysis Summary

Parameter	Reference	Method Detection Limit	Reporting Detection Limit
Microcystins by ELISA	Envirologix, KCEL SOP 440v2	0.05 µg/L(higher calibration range with MDL of 0.16 ug/L may be used for bloom samples)	0.05 µg/L
Saxitoxin by ELISA	Abraxis KCEL SOP in development	0.02 ug/L	0.02 ug/L
Cylindrospermopsin by ELISA	Beacon Analytical KCEL SOP in development	0.05 µg/L	0.05 µg/L
Anatoxin-a by HPLC	KCEL SOP #457vD	Matrix dependant, target of 0.01 – 0.25 µg/L	SPE:(MDLx2)/CF Whole Cell Analysis RDL of 5 ug/L

3.4 Laboratory Microscopic Analyses of Phytoplankton Samples

Quantitative phytoplankton identification and enumeration of cyanobacteria and other major algal groups (all taxa) will be conducted on all routine samples collected from the second June sampling through October and on the first 15 bloom scum samples. As noted above, the first June 2009 routine sampling event (30 total samples) will result in a semi-quantitative analysis for cyanobacteria taxa only with qualitative notes on other significant non-cyanobacteria taxa.

Taxonomic analysis will be performed on a single 1.0 mL subsample of each well-mixed lake sample using a Sedgewick-Rafter counting chamber (1 mL volume) and Leitz compound microscope (@100X, 400X magnification). For routinely processed samples, a transect counting methodology will be used in which successive horizontal sweeps of the full length of the S-R chamber are made under 100X power so that the entire 1 mL subsample volume is analyzed. For subsamples containing high cell densities of the most common forms, (i.e., greater than 50 cells/colonies within one transect pass), at least one half of the volume of each 1 mL subsample will be counted. In the latter case, the entire 1 mL S-R chamber will also be analyzed to enumerate all rare and very large forms, like *Ceratium* sp. Only algal cells presumed to be alive at the time of sampling (chloroplast reasonably intact in preserved sample) will be identified and counted. Phytoplankton identifications will be made to at least genus level wherever possible. Algal densities are typically reported in natural units as numbers of cells or colonies per mL.

4 DATA QUALITY OBJECTIVES

The procedures and practices described in this study-specific SAP are designed to generate data of sufficient quality to support decision making as discussed above. Critical elements of laboratory data quality objectives are discussed in this section. Procedures to attain these data quality objectives are discussed throughout this document. In particular, Section 7.0, Quality Control Procedures, addresses many of the procedures necessary to obtain data that meet these data quality objectives.

4.1 Laboratory Precision

Laboratory precision will be assessed using laboratory duplicate QC samples. When both sample results are at or exceed the MDL, the RPD (relative percent difference) should be less than 25 %. An RPD cannot be determined unless both values are at or above the MDL because no values are reported if <MDL. Note that the MDL and the Reporting Detection Limit (RDL) are the same for the ELISA.

4.2 Field Precision

Information regarding the precision of sampling procedures will be obtained by collecting field replicates. The data user should take the information obtained by collecting field replicates into account when making decisions based on data generated under this SAP.

4.3 Bias

Bias is an indicator of the accuracy of analytical data. For this project, laboratory control samples or blank spikes, whichever are available, along with matrix spikes will be used to assess bias. Results should be within 20% of the true value or within performance based criteria.

Bias will also be assessed by the evaluation of field blank and method blank data. Analytical results for method blanks should be less than the MDL.

The use of matrix spike recovery data will provide additional information regarding method performance on actual samples. The laboratory will use professional judgment regarding assessment of data quality and any subsequent action taken as a result of matrix spike recoveries.

4.4 Representativeness

This survey is primarily designed to evaluate the presence/absence of cyanobacterial toxicity, and secondarily to estimate concentrations and geographic extent of the toxin distribution, should it be present. Representative samples will be obtained through the following practices:

- The use of generally accepted sampling procedures, and
- Subsampling within the KCEL will be conducted according to lab standard operating procedures, which are designed to obtain representative subsamples

4.4.1 Representativeness and precision of phytoplankton density estimates

Laboratory subsampling protocol for microscopic phytoplankton identification requires that each sample be well-mixed (bottle gently shaken for 30-60 seconds) to insure that each subsample taken is representative of the sample container contents. Replicate subsamples of a single phytoplankton sample selected from each sampling event set (one out of 30 samples) will be analyzed as a statistical check for counting precision of the subsampling regime.

To address natural variation in plankton communities and field sampling uncertainties, field duplicate samples of routine phytoplankton will be collected at a minimum of one routine lake site for each county lake set. These duplicates will be analyzed for sample homogeneity (representative of same phytoplankton population).

4.5 Comparability

Data comparability will be obtained through the use of standard sampling procedures and analytical methods. Additionally, adherence to the procedures and QC approach contained in this SAP will provide for comparable data throughout the duration of this project. Before making changes to sample collection, storage or analysis procedures, each must be evaluated to verify that comparability will not be compromised.

4.6 Completeness

Completeness will be evaluated by the following criteria:

- The number of usable data points compared to the projected data points as detailed in this SAP

- Compliance with the data quality criteria as presented in this section
- Compliance with specified holding times

The goal for the above criteria is to obtain 100% data completeness. However, where data are not complete, decisions regarding re-sampling and/or re-analysis will be made by a collaborative process involving both data users and data generators. These decisions will take into account the project data quality objectives as presented above.

5 DATA REDUCTION, REVIEW, AND REPORTING

5.1 Toxin data

Data reduction, review and reporting will be performed using KCEL's Laboratory Information Management System (LIMS) and associated standard operating procedures. Final laboratory data will be provided to data recipients within 30 days of sample receipt or within 30 days of the decision to analyze archived samples. Data reports will include sample location, collection date and time, parameter name, measured concentration, units, method detection limit (MDL) and reporting detection limits (RDL), if available. Data will be reported in the standard KCEL comprehensive report format.

Protocols will be worked out with the KCEL for prioritization and rapid turn around of selected samples in the event of a bloom episode that could have potential public health implications. Preliminary project data, required in the event of a bloom episode that could have potential public health implications, will be reported using KCEL Preliminary Data Reporting Form followed by final data as soon as practical.

6 PROJECT ORGANIZATION

Project team members and their responsibilities are summarized in Table 5. All team members are staff of the King County Department of Natural Resources and Parks, Water and Land Resources Division.

Table 5. Project Team Members			
Name/Telephone	**Title**	**Affiliation**	**Responsibility**
Fran Sweeney (206) 684-2382 Cell (206) 683-8410	Laboratory Project Manager/Supervisor	Environmental Laboratory	Coordination of laboratory activities, lab QA/QC and data reporting
Colin Elliott (206) 684-2343	Quality Assurance Officer	Environmental Laboratory	Overall laboratory QA/QC
Gabriela Hannach (206) 684-2301	Aquatic Toxicologist	Environmental Laboratory	Coordination of toxicity analysis; Anatoxin-a method development
Maribeth Gibbons (206) 842-9382	Phytoplankton	WATER Environmental Services Inc.	Phytoplankton ID and enumeration
Joan Hardy (360) 236-3173	Toxicologist	WA State Department of Health	Grant manager
Beth Cullen (206) 263-6242	Water Quality Planner	King County Water and Land Resources	Data Manager / Field Manager
Marissa Burghdoff (425) 388-3464 x 4639	Water Quality Analyst	Snohomish County Surface Water	Coordinate field work in Snohomish County
Lindsay Tuttle (253)798-3530	Environmental Health Specialist	Tacoma/Pierce County Health Department	Coordinate field work in Pierce County

7 QUALITY CONTROL PROCEDURES

7.1 Field Quality Control Procedures

Over the course of this project, field QC samples will be collected at the frequency listed below. It is recommended that a set of field QC samples be collected during the first sampling effort to provide an initial indication of field sampling precision and bias.

7.2 Laboratory Quality Control Procedures

KCEL is accredited by the Washington State Department of Ecology (Ecology). As a requirement of this accreditation, the lab is audited by Ecology. Additionally, KCEL participates regularly in US EPA inter-laboratory performance evaluation studies.

7.2.1 Frequency of quality control samples

The frequency of quality control samples to be analyzed at KCEL for this project is shown in Table 6. All types of QC samples may not be available for all lab analyses, and certain QC may be dropped from an instrument run in order to expedite bloom/public health samples. If different extraction techniques (SPE vs. filtration of cells) are used for a batch of samples, separate QC samples will be analyzed for each technique.

Table 6. Laboratory Quality Control Samples

Type of Quality Control Sample	Description	Frequency
Method Blank	An aliquot of clean reference matrix carried through the analytical process and used as an indicator of contamination.	1 per sample batch. Maximum sample batch size equals 20 samples or one 96-well ELISA plate.
Laboratory Control Sample (LCS)	Solution of known analyte concentration, processed through the entire analytical procedure and used as an indicator of method accuracy and precision.	1 per sample batch, as available. Maximum sample batch size equals 20 samples or one 96-well ELISA plate.
Check Standard (CS)	A solution of known analyte concentration(s) that is prepared independently from calibration standard solutions and analyzed along with the samples in the analysis sequence; used to check accuracy of the calibration and indicate between-batch precision.	1 per sample batch, as available. Maximum sample batch size equals 20 samples or one 96-well ELISA plate.
Negative Control (NC)	A solution obtained from the ELISA kit manufacturer with a confirmed microcystin concentration of zero.	1 per ELISA instrument run.
Positive Control	A standardized spike of the primary ELISA toxin, typically mid-standard curve.	1 per ELISA instrument run.

Matrix Spike/ Matrix Spike Duplicate (MS / MSD)	A MS is a second sample aliquot fortified with a known concentration of target analyte(s), and processed through the entire analytical procedure; used as an indicator of sample matrix effect on the recovery of target analyte(s). A MSD is third sample aliquot fortified with a known concentration of target analyte(s), and processed through the entire analytical procedure; used as an indicator of sample matrix effect on the recovery of target analyte(s) as well as method precision. MS / MSD used with methods where samples typically show no detectable responses, thus do not provide useful information of batch precision.	1 per sample batch. Maximum sample batch size equals 20 or one 96-well ELISA plate. Note, MS/MSD may be dropped for bloom samples due to interferences from high concentrations of toxins and necessary dilutions.
Spike Blank (SB)	Known concentration of target analyte(s) introduced to clean reference matrix, processed through the entire analytical procedure and used as an indicator of method performance.	1 per sample batch. Maximum sample batch size equals 20 samples. or one 96-well ELISA plate.
Lab Duplicate	A second aliquot of a given sample, processed concurrently and identically with the initial sample, used as an indicator of method precision.	Over the course of the project, approximately 1 per week. Note that RPDs may be of limited usefulness if <MDL.

In addition to the QC samples specified above, the QC samples will be performed on samples from this project at the frequency listed in Table 7.

Table 7. Laboratory QC Requirements

Parameter	Method Blank	Lab Duplicate RPD	Negative Control	CS % Recovery	Spike Blank	Matrix Spike
Cylindrospermopsin	<MDL	25 %	<0.05 ug/L	NA	Performance based but within 20% of true value	Performance based
Saxitoxin	<MDL	25 %	<0.02 ug/L	NA	Performance based but within 20% of true value	Performance based
Microcystins	<MDL	25 %	<0. 05 ug/L	NA	Performance based but within 20% of true value	Performance based
Anatoxin a	<MDL	25 %	NA	NA	Performance based but within 20% of true value	Performance based

Performance based QC requirements rely on the development of statistically derived control limits in absence of EPA or DOE mandated control limits for an analytical method.

7.3 Corrective Action

KCEL standard operating practice is to detect and correct analytical difficulties during sample analysis. Should the lab have difficulty in meeting the data quality objectives outlined in this SAP, the lab will work with the data user to develop and implement corrective action and to document the problem and resolution using KCEL's data anomaly form (DAF).

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